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Immune regulation by $CD4^+CD25^+$ T cells and interleukin-10 in birch pollen-allergic patients and non-allergic controls

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Summary

Background $CD4^+CD25^+$ regulatory T (Treg) cells and the cytokines IL-10 or TGF-β play key roles in the maintenance of T cell homeostasis and tolerance to infectious and non-infectious antigens such as allergens.

Objective To investigate the regulation of immune responses to birch pollen allergen compared with influenza antigen by Treg cells obtained from birch pollen-allergic patients and non-allergic controls.

Methods Peripheral blood was collected from 10 birch pollen-allergic patients and 10 non-allergic healthy controls. $CD4^+CD25^+$ and $CD4^+CD25^-$ cells isolated by magnetic-activated cell sorting were co-cultured and stimulated with birch pollen extract or influenza vaccine in the absence or presence of anti-IL-10 or soluble TGF-βRII.

Results $CD4^+CD25^+$ cells from non-allergic controls were able to suppress influenza antigen and birch pollen stimulated effector cell proliferation, whereas $CD4^+CD25^+$ cells from allergic patients suppressed influenza antigen-, but not birch pollen-stimulated proliferation. The production of Th1 cytokines, but not Th2 cytokines, was suppressed by $CD4^+CD25^+$ cells from both allergic patients and controls, upon stimulation with birch pollen extract. Neutralization of IL-10 led to significantly increased production of IFN-γ in cultures with $CD4^+CD25^-$ T effector cells. In addition, six-fold higher concentrations of TNF-α were detected after neutralization of IL-10 in both $CD4^+CD25^-$ and $CD4^+CD25^+$ cell cultures from allergic patients and controls.

Conclusion We demonstrate that the allergen-specific suppressive function of $CD4^+CD25^+$ cells from allergic patients is impaired compared with non-allergic controls. Moreover, neutralization of IL-10 enhances the production of TNF-α, suggesting counter-acting properties of IL-10 and TNF-α, where IL-10 promotes tolerance and suppression by Treg cells and TNF-α promotes inflammatory responses.

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Introduction

The immune system has developed a number of means to prevent uncontrolled expansion of T cells. Among these, T cell tolerance induced by active suppression with regulatory T (Treg) cells in the periphery plays a major role in immune responses to allergens, auto-antigens, infectious antigens and tumours. Both adaptive Treg cells induced in the periphery and CD25-expressing (IL-2 receptor α-chain) Treg cells developed in the thymus during the neonatal period are of importance [1]. Regulatory $CD4^+CD25^+$ cells are defined by high CD25-expression [2] and expression of the transcription factor FOXP3 [3], while adaptive Treg cells may or may not express FOXP3 [4]. The latter maintain their suppressive ability through secretion of the suppressive cytokines IL-10 and TGF-β [5, 6].

Allergy and allergic asthma are linked to a Th2-skewed response to inhaled allergens [7]. This may be a result of a defect in the regulation of allergen-stimulated T cells [8, 9]. A deficiency of Treg cells in individuals with IgE-mediated allergic disease has been suggested. Ling et al.

[10] reported that $CD4^+CD25^+$ cells from grass pollen-allergic patients were neither able to suppress proliferation nor secretion of IL-5 and IL-13 from grass pollen-stimulated $CD4^+CD25^-$ cells during or outside the pollen season, while $CD4^+CD25^+$ cells from non-allergic controls displayed functional suppression during the season. Other studies have shown defective suppression of grass pollen-specific proliferation, with maintained suppression of IL-4, IL-5 and IFN-γ secretion [11] or retained suppression of proliferation, but reduced suppression of birch pollen-specific IL-5 and IL-13 secretion among allergic patients [12]. Depletion of $CD4^+CD25^+$ cells from, peripheral blood mononuclear cells (PBMCs) obtained from children with outgrown milk allergy induced a strong proliferative response against the milk allergen β-lactoglobulin, suggesting an important role for $CD4^+CD25^+$ cells in the induction and maintenance of oral tolerance [13].

In addition to $CD4^+CD25^+$ Treg cells, secretion of IL-10 from allergen-specific Tr1 cells, has been shown to control allergen-specific Th2-responses in allergic patients and controls [14]. The ratio between Tr1- and effector cells can thus be critical for determining development of a healthy or an allergic immune response. In the absence of Treg cells, circulating $CD4^+CD25^-$ cells from non-allergic individuals are able to recognize and respond to allergen-specific T cell epitopes [9, 10]. Moreover, it has been shown that allergen-specific immunotherapy, the only curative treatment of allergy, increases the proportion of $CD4^+CD25^+$ cells and IL-10-secreting T cells [15, 16]. Thus, suppression of allergen-specific T cells by $CD4^+CD25^+$ cells and/or through secretion of the suppressive cytokines IL-10 and TGF-β suggests overlapping allergen-specific subsets within $CD4^+CD25^+$ cells and IL-10-secreting Tr1 cells.

Here, we investigate the effect of neutralization of IL-10 and TGF-β in co-cultures with $CD4^+CD25^+$ and $CD4^+CD25^-$ cells from birch pollen-allergic patients and non-allergic controls. We demonstrate that $CD4^+CD25^+$ cells from birch pollen allergic patients exhibit an impaired suppression of birch pollen-stimulated $CD4^+CD25^-$ cells. Furthermore, we report counter-acting properties of IL-10 and TNF-α. Neutralization of IL-10 in $CD4^+CD25^+$ and $CD4^+CD25^-$ co-cultures leads to a six-fold increase of TNF-α secretion by T cells and indicates a direct or indirect function of IL-10 to modulate pro-inflammatory responses driven by TNF-α.

Material and methods

Study population

Ten birch pollen-allergic patients (27–47 years, median 33) with allergen-specific IgE to birch pollen (4.0–39 kU/L, median 8.8 kU/L) (Pharmacia CAP System, Phadia AB, Uppsala, Sweden) and allergic symptoms participated in the study. In addition, two patients had IgE to cat (0.9 and 6 kU/L). All reported rhinoconjunctivitis and one patient reported mild asthma during the birch pollen season. The control group consisted of 10 healthy non-allergic controls (24–49 years, median 31) lacking IgE to birch pollen ($\leqslant$0.35 kU/L). Blood was obtained during October–November, outside the pollen season. None of the patients had received allergen-specific immunotherapy. Oral and inhaled corticosteroids were discontinued at least 1 month and anti-histamines 5 days before sampling. The study was approved by the local ethics committee. All participants gave their informed consent.

Stimulatory and neutralizing agents

Recombinant (r) Bet v 1 (Biomay, Vienna, Austria), birch pollen extract (Aquagen SQ; ALK, Copenhagen, Denmark), influenza antigen (Vaxigrip® vaccine; Aventis Pasteur, Lyon, France), human rIL-2 (Proleukin®,;Chiron Corporation, Emeryville, CA, USA) and monoclonal anti-CD3 (OKT3) were used as cell stimulants.

Human anti-IL-10 (Mabtech, Nacka, Sweden) and soluble TGF-β receptor II (TGF-βIIR) (R&D systems, Minneapolis, MN, USA) were used in the neutralization experiments.

Isolation of $CD4^+CD25^+$ cells

PBMC were isolated by Ficoll-Paque (GE Healthcare, Uppsala, Sweden) gradient centrifugation. $CD4^+CD25^+$ cells were isolated with activated magnetic bead sorting according to the manufacturer's protocol (Treg isolation kit; Miltenyi Biotec, Bergisch Gladback, Germany). The mean purity of the isolated $CD4^+$ was 96% (95–98%) and 81% (63–94%) for $CD4^+CD25^+$ cells. Cells in the $CD4^-$ fraction were irradiated (30 Gy) and used as antigen-presenting cells, hereafter defined as ''irradiated APCs''. Owing to limitations in the number of PBMCs recovered, $CD4^+CD25^+$ were isolated from seven out of 10 allergic patients and seven out of 10 healthy controls.

T cell cultures

Co-cultures with $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were set up as triplicates in 96-well plates in the presence of antigen and 8×10^4 autologous irradiated APCs per well. For blocking experiments, a total of 2×10^4 $CD4^+$ cells (100 μL/well) were cultured in complete medium (cRPMI), i.e. RPMI 1640 medium supplemented with 25 μg/mL gentamicin, 100 IU/mL penicillin, 100 μg/mL streptomycin, 2 mM L-Gluthamine (Gibco, Invitrogen Corporation, Paisley UK), 50 μM 2-mercapto-ethanol (Sigma Aldrich, Steinheim, Germany) and 10% (v/v) heat-inactivated bovine growth serum (Hyclone, Logan, UT, USA) in the

ratios 1 : 0 ($CD4^+CD25^+$ alone), 1 : 1 and 0 : 1 ($CD4^+CD25^-$ alone). The co-cultures were stimulated with influenza antigen (1 : 1000 dilution) or 4500 U/mL of birch pollen extract in the presence or absence of 5 μg/mL anti-IL-10 antibody or 300 ng/mL soluble TGF-βRII. To evaluate suppressive function, $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were cultured in the ratios 0 : 1, 3 : 1, 1 : 1, 1 : 3 and 0 : 1 with irradiated APCs and stimulated with 1 μg/mL plate-bound anti-CD3. Cell cultures were incubated for 6 days in a humidified atmosphere with 6% CO_2 at 37 °C.

PBMC cultures were set up in 24-well plates with 6×10^5 cells/well. Cells were stimulated with 10 μg/mL rBet v 1, 4500 U/mL birch pollen extract or influenza antigen or left unstimulated in cRPMI. Twenty units per millilitres of IL-2 was added to cultures at days 5 and 8, cells were re-stimulated with antigen in the presence of 2×10^5 irradiated autologous APCs at day 10, transferred to a 96-well plate day 12 and harvested on day 14. Supernatants were collected before 1 μCi/well [^{3}H] thymidine was added for the final 18 h of incubation. Counts per minute (c.p.m.) obtained from stimulated cells were divided by c.p.m. values from unstimulated wells and expressed as stimulation indices (SIs). SI values $\geqslant 2.0$ were considered to be positive. The Th1/Th2 cytometric bead array kit (BD Biosciences, San Jose, CA, USA) was used to analyse IL-2, IL-4, IL-5, IL-10, IFN-γ and TNF-α according to the manufacturer's instruction. The detection limit was set to 2.5 pg/mL.

Flow cytometry

$CD4^+$ and $CD4^+CD25^+$ cells were stained with anti-CD25-PE (Miltenyi Biotec), anti-CD4-FITC and isotype controls IgG1-PE and IgG1-FITC (BD Pharmingen). Intracellular staining of cytokines was performed with GolgiPlug, Perm/Wash buffer and Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's instructions. Briefly, $CD4^+CD25^+$ or $CD4^+CD25^-$ cells, together with irradiated APCs, were stimulated with birch pollen extract in the presence or absence of neutralizing anti-IL-10 antibody for 2 or 6 days. Cells were further stimulated with 50 ng/mL of PMA and 250 ng/mL of Ionomycin (Sigma Aldrich) for 4 h, the final 2 h in the presence of GolgiPlug. Cells were surface stained with anti-CD25-APC and intracellulary stained with anti-TNF-α-PE or isotype control (mouse IgG1-PE) (BD Pharmingen) before analysis on a FACSCalibur flow cytometer (BD Biosciences).

The efficiency of $CD4^+CD25^+$ cell isolation was determined in three separate control experiments by intracellular staining of FOXP3 with PE-conjugated anti-human FOXP3 antibody (clone PCH101) and rat IgG2b isotype control according to the manufacturer's instructions (eBioscience, San Diego, CA, USA). Cells were analysed on a FACSAria flow cytometer (BD Biosciences).

Quantitative real-time polymerase chain reaction

Immediately after isolation, 5×10^4 $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were lysed with RNeasy lysis buffer and frozen at −70 °C. After thawing, RNA was extracted and eluted in ddH_2O using the Qiagen RNeasy micro-kit (Qiagen, Hilden, Germany), followed by reverse transcription to cDNA with ABgene's Reverse-iT 1st Strand Synthesis kit (ABgene, Surray, UK). Previously published primer pairs for FOXP3 and house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used [17] (DNA Technology, Aarhus, Denmark). Amplification of cDNA was performed in duplicates with SYBR green supermix, in the iCycler IQ sequence detection system (Bio-Rad, Hercules, CA, USA). FOXP3-expression in isolated $CD25^+$ and $CD25^-$ fractions relative expression of GAPDH was quantified ($2^{-\Delta C_t}$) and the ratio of FOXP3 mRNA in $CD25^+$ isolated fractions compared with expression in $CD25^-$ fractions was calculated.

Statistical analysis

The Wilcoxon matched pairs test was used to analyse differences in cytokine expression and proliferation in the PBMC- and $CD4^+CD25^+$ and $CD4^+CD25^-$ co-cultures. Between-group comparisons were performed using the Mann–Whitney *U*-test. A *P*-value $\leqslant 0.05$ was considered to be significant ($^* \leqslant 0.05$ and $^{**} \leqslant 0.01$).

Results

Isolated $CD4^+CD25^+$ T cells suppress polyclonal T cell activation

$CD4^+CD25^+$ cells from allergic patients and controls were isolated from peripheral blood with magnetic-activated cell sorting based on their CD25-expression. As the technique does not allow distinction between high or intermediate expression of CD25, isolated cells may contain both regulatory and activated T cells. To determine the suppressive function of the isolated cells, $CD4^+CD25^+$ and $CD4^+CD25^-$ cells, together with irradiated APCs were co-cultured in the ratios 1 : 0, 3 : 1, 1 : 1, 1 : 3 and 0 : 1 and poly clonally stimulated with plate-bound anti-CD3. Treg cells both from allergic patients and controls were equally capable of suppressing the proliferation of $CD25^-$ effector cells, resulting in a significant and almost complete (equal to $CD4^+CD25^+$ only) suppression of proliferation at the 1 : 1 ratio compared with $CD25^-$ cells only (Fig. 1a). In addition, FOXP3-mRNA expression was measured in $CD25^+$ and $CD25^-$ fractions isolated from patients and controls and the ratio of FOXP3 expression in $CD25^+$ relative to $CD25^-$ fractions was calculated. There was no difference in FOXP3 expression ratio between allergic patients and controls ($P = 0.62$), showing that the

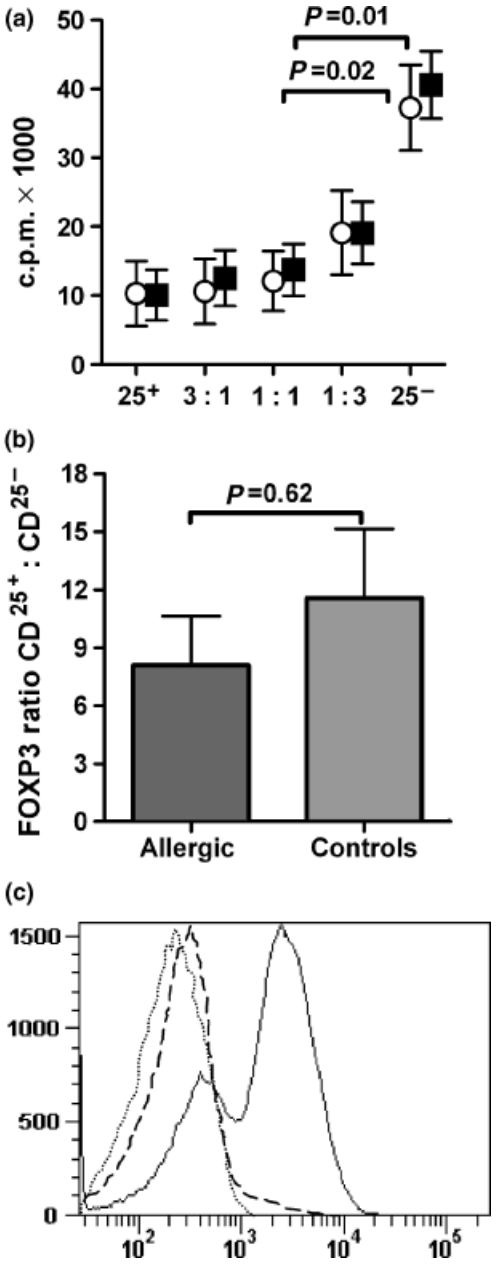

Fig. 1. Characterization of isolated $CD4^+CD25^+$ and $CD4^+CD25^-$ cell fractions. (a) Anti-CD3 stimulated proliferation of $CD4^+CD25^+$(25^+) and $CD4^+CD25^-$ (25^-) cells cultured alone and in the ratios 3 : 1, 1 : 1 and 1 : 3. Mean proliferation with standard error from seven allergic patients (○) and seven controls (■) is shown. (b) Ratio of FOXP3 mRNA expression in $CD4^+CD25^+$ cell fractions relative to $CD4^+CD25^-$ fractions, isolated from allergic patients and controls. The mean ratio with standard error is depicted in the figure. (c) Expression of FOXP3 protein in isolated $CD4^+CD25^+$(filled line), $CD4^+CD25^-$ cells (dashed line) and isotype control (dotted line). The figure represents one of three independent experiments. c.p.m., counts per minute.

sorting of $CD4^+CD25^+$ Treg cells was equally efficient for both groups (Fig. 1b). The method of isolation was further evaluated by measuring intracellular FOXP3 protein in isolated $CD4^+CD25^+$ and $CD4^+CD25^-$ cells from three individuals. The results showed that typically 68% (63–73%) of the $CD4^+CD25^+$ cells and 6.3% (5–7%) of $CD4^+CD25^-$ contained FOXP3 (Fig. 1c). Collectively, these data support the fact that the majority of the isolated $CD4^+CD25^+$ cells were FOXP3+ Treg cells with equal suppressive function whether isolated from allergic patients or healthy controls.

Allergen-specific proliferation by peripheral blood mononuclear cells from birch pollen-allergic patients

PBMCs from 10 birch pollen-allergic patients and 10 healthy controls were stimulated with birch pollen extract, rBet v 1 (the major birch pollen allergen) and influenza antigen. PBMCs from birch pollen-allergic patients proliferated upon stimulation with birch pollen extract and rBet v 1, compared with PBMCs from non-allergic controls, who did not exhibit a positive proliferation ($P = 0.027$ and 0.017, respectively). Median SI for the birch pollen-allergic patients was 3.3 (1.4–13.5) for stimulation with rBet v 1, compared with 1.5 (0.5–6.7) for healthy controls, and for stimulation with birch pollen extract 4.6 (0.2–10.3) for patients and 1.2 (0.2–20.1) for controls. Influenza antigen induced proliferation by PBMCs from both patients and controls. The SI values after stimulation with influenza antigen did not differ significantly between allergic patients, median SI 3.1 (1.2–6.1) and non-allergic controls, 4.5 (0.8–44.8) (Fig. 2a).

Dysfunctional allergen-specific suppression by $CD4^+CD25^+$ T cells in allergic individuals

Having established that the isolated $CD4^+CD25^+$ cells contain a majority of functional Treg cells, we sought to determine their ability to suppress antigen-specific responses. First, we evaluated whether Treg cells from seven allergic patients and seven non-allergic controls were able to suppress $CD25^-$ effector cells stimulated with influenza antigen or birch pollen extract. No difference in the ability of $CD4^+CD25^+$ cells from allergic patients and healthy controls to suppress proliferation of influenza antigen-stimulated $CD4^+CD25^-$ cells was observed (Fig. 2b, significant suppression for both groups, $P = 0.017$). However, $CD4^+CD25^+$ from birch pollen-allergic patients did not suppress birch pollen extract-stimulated $CD4^+CD25^-$ effector cells as efficiently as cells from non-allergic controls ($P = 0.017$) (Fig. 2c).

Second, we investigated cytokine production by $CD4^+CD25^+$ and $CD4^+CD25^-$ cells from allergic patients and controls (Fig. 3). Upon stimulation with influenza antigen, $CD4^+CD25^-$ effector cells from allergic patients and non-allergic controls produced IL-2, IL-4, IL-5, IL-10, IFN-γ and TNF-α. $CD4^+CD25^+$ cells from non-allergic controls were able to suppress production of all detected

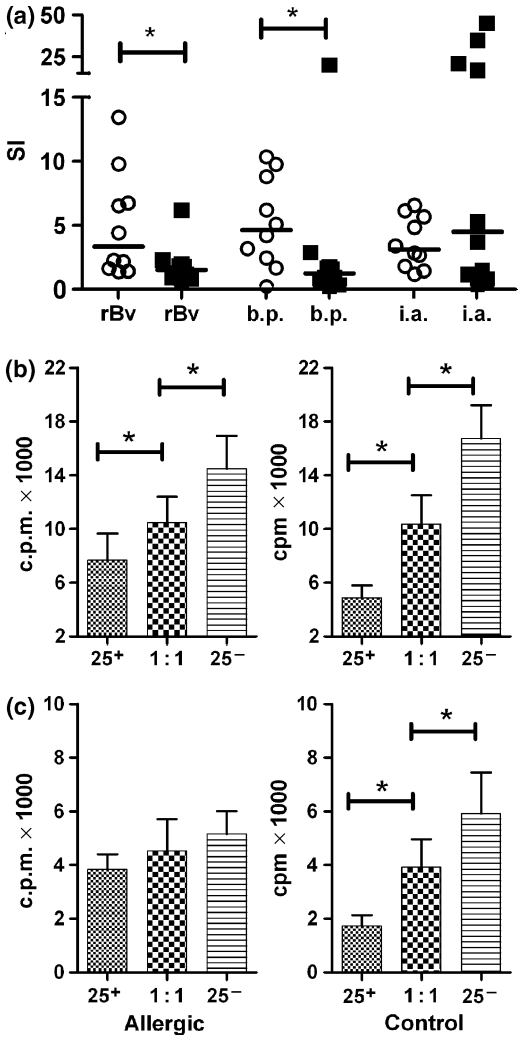

Fig. 2. Proliferation of peripheral blood mononuclear cells (PBMCs) and isolated $CD4^+CD25^+$ and $CD4^+CD25^-$ cells from birch pollen-allergic patients and non-allergic controls. (a) Proliferation of PBMCs from 10 allergic patients (○) and 10 non-allergic controls (■) upon stimulation with rBet v 1 (rBv), birch pollen extract (b.p.) or influenza antigen (i.a.). Horizontal lines represent median SI. (b and c) $CD4^+CD25^+(25^+)$ and $CD4^+CD25^-$ cells (25^-) from seven birch pollen-allergic patients and seven non-allergic controls were cultured alone or in a one-to-one ratio (1 : 1) and stimulated with influenza antigen (b) or birch pollen extract (c). The mean counts per minute (c.p.m.) values with standard error are shown. $^*P \leqslant 0.05$. SI, stimulation index

cytokines ($P = 0.017$), except IL-10, while $CD4^+CD25^+$ from birch pollen-allergic patients suppressed influenza antigen-induced secretion of IL-2, IFN-γ ($P = 0.017$) and TNF-α (NS $P = 0.062$), but not IL-4, IL-5 and IL-10 (1 : 1 $CD25^+ : CD25^-$ co-cultures compared with $CD25^-$ effector cells). A tendency was noted for $CD4^+CD25^+$ cells from some allergic donors to suppress influenza antigen induced IL-4 production by $CD4^+CD25^-$ cells (NS $P = 0.10$). Birch pollen extract-stimulated production of IL-2, IFN-γ and TNF-α was suppressed by $CD4^+CD25^+$ cells from both allergic patients and healthy controls ($P = 0.017$). Production of IL-4 and IL-5 by $CD4^+CD25^-$ cells was not suppressed by the $CD4^+CD25^+$ cells either from allergic patients or controls. Significantly more IL-5 was detected in wells with $CD4^+CD25^+$ cells alone and 1 : 1 $CD25^+ : CD25^-$ co-cultures from allergic patients compared with controls ($P = 0.042$ and 0.036, respectively). There was no difference in IL-4 secretion between groups.

IL-10 production by $CD4^+CD25^-$ cells was not subjected to suppression; in fact, upon allergen stimulation, $CD4^+CD25^+$ cells from allergic patients produced more IL-10 compared with $CD4^+CD25^-$ cells ($P = 0.027$). Similar results were obtained in $CD4^+CD25^+$ cell cultures from healthy controls, but the difference compared with $CD4^+CD25^-$ cultures did not reach statistical significance ($P = 0.062$). Taken together, the cytokine results indicated that Treg cells from non-allergic controls suppressed influenza antigen-stimulated production of Th1 cytokines IFN-γ and TNF-α to the same extent as Th2 cytokines, represented here by IL-4 and IL-5, while Treg cells from allergic patients merely suppressed Th1 cytokines. Stimulation with birch pollen extract resulted in suppressed production of Th1 but not Th2 cytokines in 1 : 1 co-cultures of $CD4^+CD25^+$ and $CD4^+CD25^-$ cells from both allergic individuals and non-allergic controls.

Neutralization of interleukin-10 and transforming growth factor-β in co-cultures with $CD4^+CD25^+$ and $CD4^+CD25^-$ cells

To address the question of whether the suppressive cytokines IL-10 or TGF-β are important for Treg cell function following allergen exposure, we stimulated $CD4^+CD25^+$ and $CD4^+CD25^-$ cells from allergic patients and non-allergic controls, with birch pollen extract in the presence or absence of neutralizing anti-IL-10 antibody or soluble TGF-βRII. Neutralization of IL-10 or TGF-β did not affect influenza- or birch pollen-stimulated proliferation (Fig. 4a, influenza-stimulated proliferation not shown). Analysis of secreted cytokines revealed that addition of soluble TGF-β receptor increased IFN-γ production by effector cells obtained from healthy controls ($P = 0.027$) and neutralization of IL-10 increased the production of IFN-γ by effector cells both from healthy controls ($P = 0.027$) and allergic patients ($P = 0.017$), and also by $CD4^+CD25^+$ cells from allergic patients ($P = 0.017$) (Fig. 4b). Interestingly, neutralization of IL-10 induced a six-fold increase of the production of TNF-α both when $CD4^+CD25^+$ cells and $CD4^+CD25^-$ cells were cultured separately and in co-cultures at a one-to-one ratio (Fig. 5). The strong TNF-α production in the absence of IL-10 compared with cultures without anti-IL-10 antibody was detected both for allergic patients ($P = 0.027$) and controls ($P = 0.027$) but was more pronounced for cells from allergic patients (NS). The median values of TNF-α concentrations in $CD4^+CD25^+$

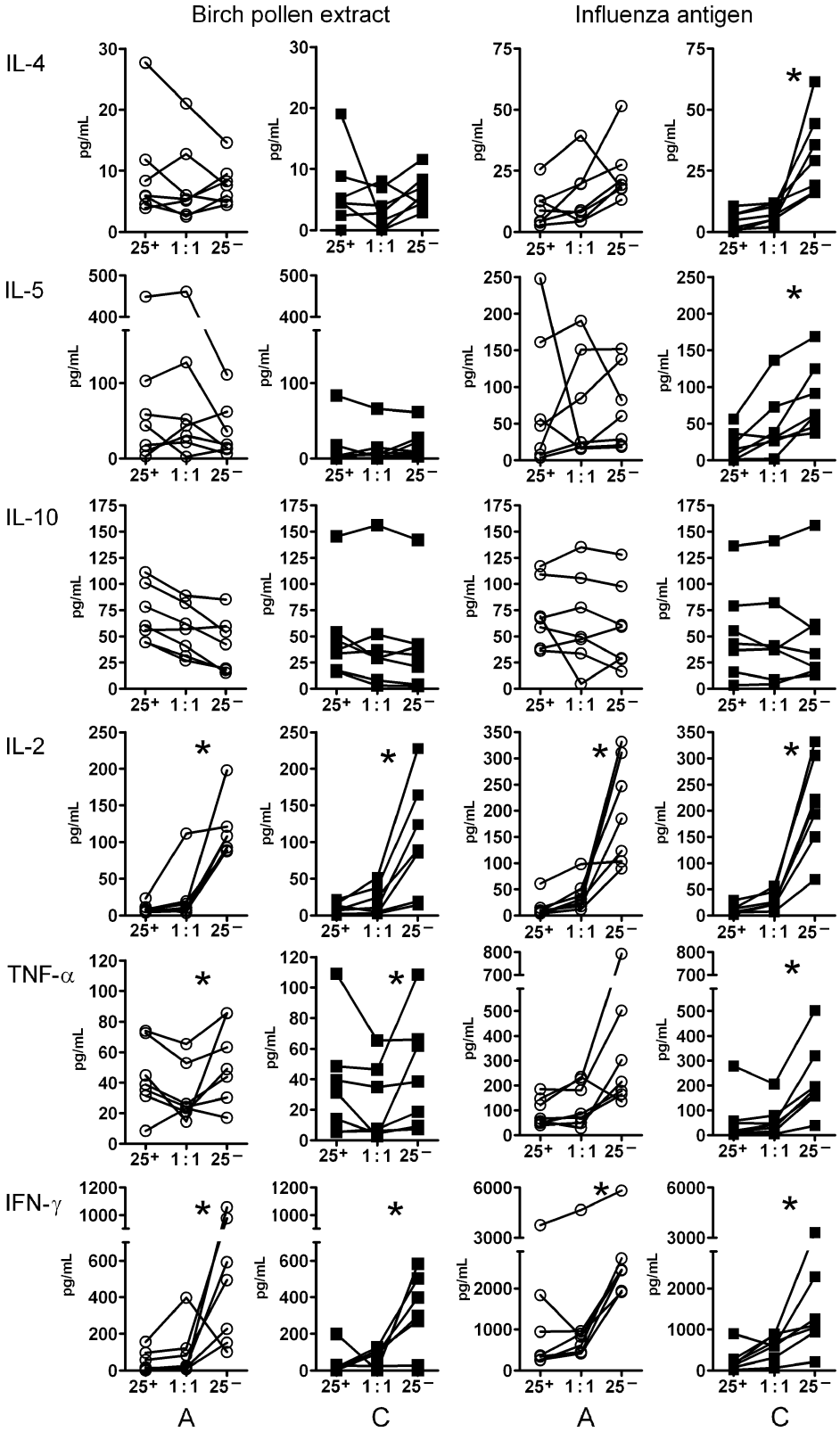

Fig. 3. Cytokine production in co-cultures with $CD4^+CD25^+$ and $CD4^+CD25^-$ cells stimulated with birch pollen extract and influenza antigen. IL-4, IL-5, IL-10, IL-2, TNF-α and IFN-γ concentrations (pg/mL) were measured in supernatants collected from $CD4^+CD25^+$(25^+) and $CD4^+CD25^-$ (25^-) cells cultured alone or in a one-to-one ratio (1 : 1) from allergic patients A (○) and controls C (■). Columns one and two represent cultures stimulated with birch pollen extract and columns three and four influenza antigen, as indicated. $^*P \leqslant 0.05$.

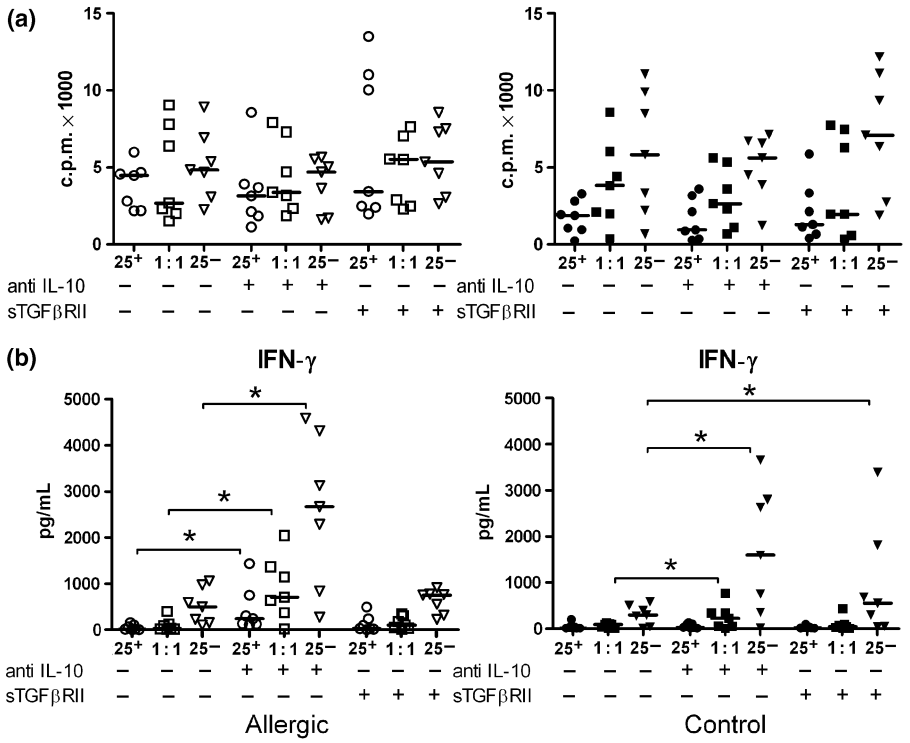

Fig. 4. Neutralization of IL-10 and TGF-β in co-cultures with $CD4^+CD25^+$ and $CD4^+CD25^-$ cells stimulated with birch pollen extract. Proliferation expressed as counts per minute (c.p.m.) values (a) and production of IFN-γ as concentration (pg/mL) in cell supernatants (b) in co-cultures with $CD4^+CD25^+(25^+)$ and $CD4^+CD25^-$ (25^-) cells from birch pollen-allergic patients (open symbols) and healthy controls (filled symbols). Cells were cultured ± anti-IL-10 or sTGF-βRII and stimulated with birch pollen extract. For comparison, the proliferation and IFN-γ data from co-cultures without a neutralizing agent also depicted in Figs 2 and 3 respectively are shown. Horizontal lines represent median concentrations. $^*P \leqslant 0.05$.

cultures from allergic patients and healthy controls without neutralizing anti-IL-10 antibody were 38.9 (8.4–74.0) and 31.2 (6.0–108.1) pg/mL, respectively, and 292.0 (115.3–720.4) and 138.1 (5.3–367.8) pg/mL in the presence of neutralizing antibody. For $CD4^+CD25^-$ cells, the median TNF-α values without blocking antibody were 48.9 (17.8–85.6) pg/mL for allergic patients and 38.4 (7.3–108.1) pg/mL for healthy controls. In the presence of neutralizing anti-IL-10 antibody, the $CD4^+CD25^-$ cells produced 313.2 (31.1–554.2) and 140.9 (7.9–388.6) pg/mL, respectively. Addition of neutralizing antibody to IL-10 completely abolished (below 2.5 pg/mL) detectable IL-10 in the supernatants. Thus, neutralization of IL-10 led to increased production of TNF-α that was not dependent on allergy, nor was it confined to one of the $CD4^+CD25^+$ or $CD4^+CD25^-$ cell fractions (Fig. 5).

In order to ensure that irradiated APCs were not responsible for the production of TNF-α, intracellular staining of TNF-α in $CD4^+CD25^+$, $CD4^+CD25^-$ and irradiated APCs were performed after 2 and 6 days of incubation with birch pollen extract in the presence of neutralizing anti-IL-10 antibody. On days 2 and 6, 2.2% and 0.3% of the irradiated APCs produced TNF-α, compared with $CD4^+CD25^+$ and $CD4^+CD25^-$ cells, where 16.1% and 12.9% of the cells produced TNF-α day 2 and 23.1% and 19.5% day 6. Thus $CD4^+$ cells were indeed the main producers of TNF-α. In addition, neutralization of IL-10 induced up-regulation of CD25 on $CD4^+CD25^-$ effector cells. The CD25-expression increased in average 1.8% (0.8–3.3%) compared with cultured cells without neutralizing antibody in three independent experiments (not shown).

Discussion

Peripheral T cell tolerance induced by Treg cells is of major importance for the immune systems' responses to allergens. We demonstrate that $CD25^+$ Treg cells from birch pollen-allergic patients display an impaired ability to suppress birch pollen stimulated proliferation of $CD25^-$ effector cells. Our results suggest that circulating Treg cells in non-allergic individuals suppress proliferation of allergen-specific effector cells upon exposure to allergens, while Treg cells from sensitized individuals are not able to suppress allergen-specific effector cells to the same extent, in agreement with previously published reports [8, 10]. However, inconsistent results have been reported on whether suppression of proliferation or Th2 cytokines or both is dysfunctional in allergic individuals [10–12]. In our experiments, Treg cells from allergic

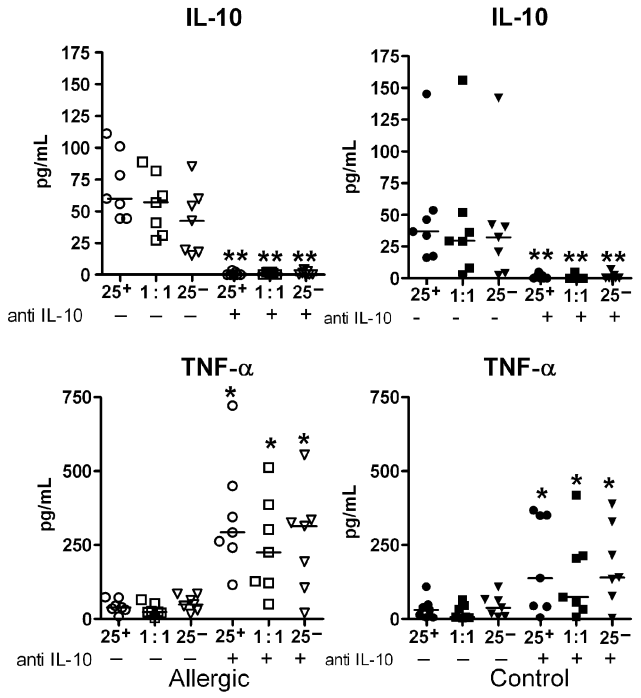

Fig. 5. TNF-α production in co-cultures with $CD4^+CD25^+$ and $CD4^+CD25^-$ cells after neutralization of IL-10. $CD4^+CD25^+(25^+)$ and $CD4^+CD25^-$ (25^-) cells from birch pollen-allergic patients (open symbols) and non-allergic controls (filled symbols) were cultured alone and in a one-to-one ratio $(1:1) \pm$ anti-IL-10 and stimulated with birch pollen extract. Data for production of IL-10 and TNF-α in co-cultures without anti-IL-10 also depicted in Fig. 3 are given for comparison. Horizontal lines represent median concentrations. $^*P \leqslant 0.05$, $^{**}P \leqslant 0.01$.

patients did not suppress birch pollen extract or influenza antigen-stimulated secretion of IL-4 or IL-5. In comparison, Treg cells from controls were able to suppress the secretion of IL-4 and IL-5 upon stimulation with influenza antigen but not upon stimulation with birch pollen extract. However, the birch pollen-induced production of IL-4 and IL-5 among cells from non-allergic donors was low compared with influenza-stimulated cytokine production, making it difficult to interpret the ability of Treg cells to suppress allergen-stimulated IL-4 and IL-5. It has previously been shown that Th2 cells are less sensitive than Th1 cells to the suppressive activity of $CD25^+$ Treg cells indicating different mechanisms for suppression of Th1 and Th2 responses [18, 19]. Consequently, the differences between capability to suppress influenza-stimulated secretion of Th2 cytokines by Treg cells from allergic patients and non-allergic controls found in our study could be explained by a more general Th2 pre-disposed immune response in allergic individuals [20, 21].

It has been suggested that TGF-β is necessary for the Treg cell function in murine systems [22, 23]. Our results are not inline with those data, as neutralization of TGF-β did not influence suppressive function or cytokine production by Treg cells. Yet, Treg cell suppression *in vitro* may differ from the mechanism of suppression *in vivo* [24]. Neutralization of IL-10 led to increased secretion of IFN-γ and TNF-α, without affecting cell proliferation. Thus, the observed increase in cytokine production is not a result of increased cell numbers but rather of released suppression of cytokine expression in the absence of IL-10. According to our data, IL-10 specifically suppresses IFN-γ production by $CD25^-$ effector cells. In contrast, secretion of TNF-α increased significantly both from Treg and effector cells in cultures with neutralized IL-10 compared with cultures without blocking antibody. Neutralization of IL-10, in LPS-stimulated cultures with placenta cells, increased the secretion of TNF-α, while addition of exogenous IL-10 decreased the TNF-α secretion [25], which indicates an important role for IL-10 in regulation of TNF-α secretion. TNF-α is a pro-inflammatory cytokine involved in the pathogenesis of inflammatory diseases such as rheumatoid arthritis [26] and asthma [27, 28]. Lately, an antagonist to TNF-α has been administered to patients with asthma, resulting in reduced expression of membrane-bound TNF-α, improved airway function [29] and reduced airway hyperresponsiveness [27]. Moreover, recent reports suggest that anti-TNF treatment increases the number of $CD25^+$ Treg cells as well as restores suppressive function of these cells [26, 30]. Valencia et al. showed that TNF-α directly inhibits the suppressive function of Treg cells. The effect of TNF-α is concentration dependent and mediated through signalling and up-regulation of TNFRII, which leads to a decreased expression of FOXP3. The increased levels of TNF-α during infections and chronic inflammation were thus proposed to down-regulate FOXP3 and inhibit the function of Treg cells [26].

As complete neutralization of IL-10 in $CD25^+$ Treg cells, $CD4^+CD25^-$ cells and co-cultures stimulated with birch pollen extract corresponds to elevated levels of TNF-α, it is tempting to speculate that secretion of IL-10, by the Treg cells themselves or by other cell types, maintains homeostasis and function of Treg cells in the periphery under normal conditions. Indeed, allergen-specific immunotherapy, with a successful clinical outcome, involves increased IL-10 secretion [8, 16, 31, 32]. Noteworthy, we demonstrate that similar amounts of IL-10 were detected in supernatants of $CD4^+CD25^+$ and $CD4^+CD25^-$ cells both from allergic patients and controls after stimulation with birch pollen extract. Allergic donors' $CD25^+$ cells produced even more IL-10 than the $CD25^-$ cell fraction, indicating that IL-10-secreting cells are present in both T cell subsets. Possibly, the IL-10 production can be induced by $CD25^+$ Treg cells as shown in a mouse allergy model [33]. Moreover, neutralization of IL-10 induced up-regulation of CD25 on $CD4^+CD25^-$ cells. The induction of CD25-expression may be due to activation of antigen specific $CD25^-$ effector cells in the absence of IL-10. The molecular mechanism of how IL-10 counter-acts secretion

of TNF-α is not fully understood [34]. It is possible that TNF-α itself may modulate the suppressive function and secretion of IL-10. Iwasaki et al. [35] reported that ovalbumin (OVA)-sensitized TNF-α knock-out mice had fewer allergic symptoms, decreased amount of OVA-specific IgE and reduced IL-10 production compared with wild-type mice implying an interaction between IL-10 and TNF-α in allergic disease models. In humans, OX40L has been proposed to act as a switch that converts regulatory IL-10-producing Th1 or Th2 cells into TNF-α-producing inflammatory cells in the absence of IL-12 [36]. Furthermore, murine T cells subjected to a fusion protein blocking the interaction between OX40 and its ligand *in vivo* produced less TNF-α compared with control mice [37].

In conclusion, we demonstrate that Treg cells from birch pollen-allergic patients display an impaired ability to suppress birch pollen-stimulated effector cells. In addition, neutralization of IL-10 in $CD25^+$ Treg cell and $CD25^-$ effector cell co-cultures induces a significant increase of TNF-α secretion. We propose that IL-10 and TNF-α may have counter-acting properties in the periphery, where IL-10 promotes tolerance and suppression by Treg cells and TNF-α promotes inflammatory responses.

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